UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

CÉSAR ANDRÉS RIVERA MARTÍNEZ

PROGNOSTIC BIOMARKERS AND ROLE OF AGRIN IN PROGRESSION OF ORAL CANCER

BIOMARCADORES PROGNÓSTICOS E PAPEL DE AGRINA NA PROGRESSÃO DO CÂNCER ORAL

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Medicine and Oral Pathology, in the Stomatology area.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Estomatopatologia, na Área de Estomatologia.

Orientador: Profa. Dra. Adriana Franco Paes Leme

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL
DA TESE DEFENDIDA PELO ALUNO CÉSAR ANDRÉS
RIVERA MARTÍNEZ E ORIENTADA PELA PROFA.
DRA. ADRIANA FRANCO PAES LEME.

Piracicaba
2017

Agência(s) de fomento e nº(s) de processo(s): CONICYT, 8540/2014
ORCID: http://orcid.org/0000-0002-5491-4233

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

R524p

Rivera Martínez, César Andrés, 1983-
Prognostic biomarkers and role of agrin in progression of oral cancer / César Andrés Rivera Martínez. – Piracicaba, SP : [s.n.], 2017.

Orientador: Adriana Franco Paes Leme.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Neoplasias bucais. 2. Carcinoma de células escamosas. 3. Biomarcadores tumorais. 4. Agrina. 5. Proteômica. I. Leme, Adriana Franco Paes. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Biomarcadores prognósticos e papel de agrina na progressão do câncer oral
Palavras-chave em inglês:
Mouth neoplasms
Carcinoma, squamous cell
Tumor biomarkers
Agrin
Proteomics
Área de concentração: Estomatologia
Titulação: Doutor em Estomatopatologia
Banca examinadora:
Adriana Franco Paes Leme [Orientador]
Bernardo Arturo Venegas Rojas
André Zelanis Palitot Pereira
Fabio Albuquerque Marchi
Alan Roger dos Santos Silva
Data de defesa: 06-11-2017
Programa de Pós-Graduação: Estomatopatologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 06 de Novembro de 2017, considerou o candidato CÉSAR ANDRÉS RIVERA MARTÍNEZ aprovado.

PROFª. DRª. ADRIANA FRANCO PAES LEME

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PROF. DR. FABIO ALBUQUERQUE MARCHI

PROF. DR. ALAN ROGER DOS SANTOS SILVA

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATION

This thesis work is dedicated to my wife, María Jesús, who has been a constant source of love, support and encouragement. I am truly thankful for having you in my life. This work is also dedicated to my parents, Angélica and César, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

ACKNOWLEDGMENTS

I would like to thank all the people who contributed in some way to the work described in this thesis.

Foremost, I would like to express my sincere gratitude to my advisor Prof. Dr. Adriana Franco Paes Leme for the continuous support of my Ph.D study and research, for her motivation, enthusiasm and knowledge. Her guidance helped me in all the time of research and writing of this thesis.

My sincere thanks also goes to University of Talca (Chile), my alma mater, who supported me from the outset. I will work, sow and reap!

I would like to thank to the postgraduate program in Oral Medicine and Oral Pathology (FOP/UNICAMP), for giving me certainties and questions that will accompany me forever.

I thank my fellow labmates in the MAS Group, in particular Flávia, Rute, Tati, Ana Karina, Ariane, Carol CS Macedo, Carol M Carnielli, Jamile and Rachel for the stimulating discussions, and for all the fun we have had in the last years.

Thanks also to Bianca, Dani, Romênia and Sami, members of the MAS group.

Furthermore, I would like to thank my colleagues Daniel, Wilfredo, Celeste and Ciro for their support in the histopathological tissue analysis.

I am grateful to Dr. Flavia Vischi Winck for enlightening me the first glance of research.

Also I thank all my Chilean and Brazilian friends in Campinas.

Last but not the least, I would like to thank my wife María Jesús and my parents Angélica and César, for encourage me to fly toward my dreams.

"I discovered the secret of the sea in meditation upon a dewdrop."

Khalil Gibran, 1883-1931.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck. It represents the most prominent malignant neoplasm for dental surgeons. It is usually detected in advanced clinical stages, and despite the medical advances, patients still have a poor clinical prognosis. It justifies the search for prognostic biomarkers that represent the staging of the disease, as well as understand molecular mechanisms that control its physiopathology. The objective of this doctoral thesis was to identify the prognostic biomarkers provided in biomedical literature, as well as to deepen the understanding of the role of a proteoglycan named agrin. Agrin proved to be relevant in several oncogenic events in previous studies published by our group. To achieve our first objective, we performed a systematic review of the literature. In this study, we used the MEDLINE/PubMed database and keywords associated with patient risk and common OSCC clinical endpoints: overall survival, disease-free survival and cause-specific survival. This approach produced an article that identified 41 potential prognostic biomarkers, mainly proteins evaluated by immunohistochemistry. These potential biomarkers must be clinically evaluated in new clinical studies. For the second aim of this thesis, we silenced the expression of agrin (using shRNA technology) in different cell lines and identified the protein network associated with agrin through mass spectrometry-based proteomics. We assessed the prognostic value of this network using bioinformatics tools and public databases. Our results suggest that agrin is essential for the oncogenic events associated with the progression of the OSCC, both *in vivo* and *in vitro*, which led us to conclude that agrin is a strong candidate as a therapeutic target for OSCC.

Key Words: Mouth neoplasms. Carcinoma, squamous cell. Tumor biomarkers. Agrin. Proteomics.

RESUMO

O carcinoma de células escamosas da cavidade oral (CECO) é o câncer mais comum na região da cabeça e pescoço. O CECO representa a neoplasia maligna mais importante para os cirurgiões dentistas. É detectado geralmente em estágios clínicos avançados e, apesar dos progressos da medicina, os pacientes ainda apresentam um prognóstico clínico desfavorável. Esse contexto justifica a busca de biomarcadores prognósticos que representem o estadiamento da doença, além de compreender os mecanismos moleculares que controlam sua fisiopatologia. Os objetivos desta tese de doutorado foram identificar os potenciais biomarcadores prognósticos para CECO descritos na literatura biomédica, bem como aprofundar a compreensão do papel do proteoglicano agrina. Essa proteína mostrou-se relevante em vários eventos oncogênicos em estudos prévios publicados por nosso grupo. Para alcançar nosso primeiro objetivo, realizamos uma revisão sistemática da literatura. Nesse estudo, utilizamos a base de dados MEDLINE/PubMed e palavras-chave associando o risco dos pacientes com os seguintes desfechos clínicos para CECO: sobrevida global, sobrevida livre de doença e sobrevida doença-específica. Essa abordagem resultou na publicação de um artigo que identificou 41 potenciais biomarcadores prognósticos em CECO, principalmente proteínas avaliadas por inmunohistoquímica. Esses potenciais biomarcadores devem ser avaliados em novos estudos clínicos. Para o segundo objetivo desta tese, silenciamos a expressão de agrina (utilizando a tecnologia shRNA) em diferentes linhagens celulares e identificamos a rede de proteínas associadas a agrina através de proteômica baseada em espectrometria de massas. O valor prognóstico desta rede foi avaliado utilizando ferramentas de bioinformática e bancos de dados públicos. Nossos resultados sugerem que a proteína agrina é essencial para os eventos oncogênicos associados à progressão do CECO, tanto *in vivo* como *in vitro*, o que nos leva a concluir que agrina é um forte candidato como alvo terapêutico para o CECO.

Palavras-chave: Neoplasias bucais. Carcinoma de células escamosas. Biomarcadores tumorais. Agrina. Proteômica.

LIST OF ABBREVIATIONS AND ACRONYMS

OSCC	-	Oral squamous cell carcinoma.
shRNA	-	Short hairpin RNA or small hairpin RNA.
CEC	-	Carcinoma de células escamosas da cavidade oral.
UTALCA	-	University of Talca (Chile).
CEUA	-	Institutional Animal Ethics Committee.
CNPEM	-	Brazilian National Center for Research in Energy and Materials.
HPV	-	Human papillomavirus.
HNSCC	-	Head and neck squamous cell carcinoma.
NCCN	-	National Comprehensive Cancer Network.
ECM	-	Extracellular matrix.
HSPGs	-	Heparan sulfate proteoglycans.
TNM	-	Classification of malignant tumors. T, size of the original (primary) tumour. N, nearby (regional) lymph nodes that are involved. M, distant metastasis.
REMARK	-	REporting recommendations for tumor MARKer prognostic studies.
NCI	-	National Cancer Institute (USA).
OS	-	Overall survival.
DFS	-	Disease-free survival. Also called relapse-free survival.
CSS	-	Cause-specific survival.
MeSH	-	Medical Subject Headings.
RR	-	Risk ratio or relative risk.
OR	-	Odds ratio.
HR	-	Hazard ratio.
PRISMA	-	Preferred Reporting Items for Systematic Reviews and Meta-Analyses.
IHC	-	Immunohistochemistry.
WHO	-	World Health Organization.
TCGA	-	The Cancer Genome Atlas.
CONICYT	-	Chilean National Commission for Scientific and Technological Research.
PNPD	-	Brazilian National Program for Post-Doctorates.
CAPES	-	Brazilian Federal Agency for Post-graduate Education.

FAPESP	-	São Paulo Research Foundation.
RT-qPCR	-	Real-time quantitative polymerase chain reaction.
FISH	-	Fluorescence in situ hybridization.
HGNC	-	HUGO (Human Genome Organization) Gene Nomenclature Committee.
NS	-	Not specified.
NA	-	Not apply.
Ct-agrin	-	C-terminal fragment of agrin. Also C-agrin$_{4,19}$-GFP construct.
NOD-SCID	-	Nonobese diabetic/severe combined immunodeficiency.
FMUSP	-	Faculty of Medicine, University of São Paulo.
shAgrin	-	Agrin silenced cells.
shControl	-	Non-silencing control cells.
GFP	-	Green fluorescent protein.
Ip-control	-	FLAG-tagged GFP vector.
TBST	-	Or TTBS. Mixture of tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20).
SDS	-	Sodium dodecyl sulfate.
SDS–PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
PBS	-	Phosphate-buffered saline.
IPAD	-	Pathway Analysis Database for Systematic Enrichment Analysis.
CHAT	-	Contextual Hub Analysis Tool.
STRING	-	Search Tool for the Retrieval of Interacting Genes.
EMT	-	Epithelial-mesenchymal transition.

CONTENTS

1 INTRODUCTION

Oral cancer is a highly relevant problem of global public health, especially for dentistry. Despite the progress in research and therapy, survival has not improved significantly in the last years, representing a continuing challenge for biomedical sciences.

Oral cancer is a malignant neoplasia which arises on the lip or oral cavity. It is traditionally defined as a squamous cell carcinoma (OSCC) (Rivera, 2015). OSCC represents 95% of all forms of head and neck squamous cell carcinoma (HNSCC) (Rivera and Venegas, 2014), constituting the most common malignancy of head and neck region (Chi et al., 2015). Oral cancer is two to three times more prevalent in men than women in most ethnic groups. In worldwide reports, cancers of all regions of the oral cavity and pharynx are grouped and collectively represent the sixth most common cancer in the world (Rivera, 2015).

OSCC is a preventable disease, where smoking and alcohol-considered major risk factors-are present in 90% of cases (Dissanayaka et al., 2012), having both a synergic effect (Koontongkaew, 2013). Among other risk factors, there is the human papillomavirus (HPV, mainly associated with nonkeratinizing squamous cell carcinoma of the oropharynx) (Dalianis, 2014) and ultraviolet radiation (associated with carcinoma of lips)(Gallagher et al., 2010).

OSCC can be presented as a “natural history”, which originates from non-aberrant keratinocytes which are chronically exposed to a stimulus that breaks its homeostasis, following an epithelial hyperplasia, dysplasia in different degrees, carcinoma in situ and an invasive carcinoma leading to the generation of distant metastases, with the consequent clinical manifestations (Rivera, 2015).

Despite the fact that cancer occurs in a part of the body that is readily accessible for early detection, most carcinomas are not diagnosed until they have reached advanced stages (Jafari et al., 2013). In addition, therapeutic alternatives (surgery, radiation and chemotherapy) remain highly expensive and disfiguring (CDC, 2013). These precedents justify the search and need for OSCC biomarkers.

The term biomarker refers to a measurement variable that is associated with disease outcome (Ballman, 2015). They can be used for patient assessment in multiple clinical scenarios, including estimating the risk of disease and distinguishing benign from malignant lesions (Henry and Hayes, 2012). Tumor markers are mostly useful in evaluating the progression of the disease status (Kabel, 2017). They can be classified in predictive, diagnostic and prognostic biomarkers (Mishra and Verma, 2010).

Prognostic tumor markers aim to objectively evaluate the patient's cancer outcome (e.g., overall survival, disease-free survival, and cause-specific survival) independent of treatment received (Ballman, 2015). The presence or absence of a prognostic marker can be useful for the selection of a better therapeutic regimen (Mehta et al., 2010).

Today, it is accepted that HPV status is a HNSCC biomarker. HPV-associated HNSCCs (nonkeratinizing) form a distinct clinical entity with favorable outcomes (Dok and Nuyts, 2016; TCGA, 2015). The current National Comprehensive Cancer Network (NCCN) guidelines state that "HPV testing is valuable prognostically; however, the results should not change management decisions" (Adelstein et al., 2017). Therefore, it is necessary to identify other biomarkers that can assist in these decisions.

In addition to HPV, several biomarkers have been suggested to foresee the prognosis of OSCC patients. Knowing who the prognostic OSCC biomarkers are may be the first step to their arrival at the clinic.

A key to the development of more effective therapy lies in a better understanding of the mechanisms involved in the progression of OSCC (Malik et al., 2016). Tumors are encircled by extracellular matrix (ECM) (Wang et al., 2017). The ECM modulates the hallmarks of cancer, and changes in its dynamics contribute to tumor progression (Pickup et al., 2014). Some components of the ECM, which include heparan sulfate proteoglycans (HSPGs), are frequently overproduced in cancer (Lu et al., 2012). These molecules have a clinical potential for HNSCC (Farnedi et al., 2015).

In a previous study, our group demonstrated that a HSPG called agrin induces OSCC cell adhesion and migration (Kawahara et al., 2014). In addition, agrin has been shown to act as a sensor in developing tumorigenic signals associated with the ECM in hepatocellular carcinomas (Chakraborty et al., 2015). These results suggested that agrin has an oncogenic role in oral cancer, furthermore, in the context of OSCC progression, there are no studies evaluating the role of agrin.

In view of OSCC challenges set out in the content of this introduction, this doctoral thesis is aimed to i) identify, evaluate and summarize the evidence for reported prognostic biomarkers and ii) examine the role of agrin in the progression of this disease.

This text was carried out in the alternative format, according to the Central Postgraduate Commission (CCPG/001/2015), University of Campinas.

2 ARTICLES

2.1 Prognostic biomarkers in oral squamous cell carcinoma: a systematic review. Oral Oncology. Volume 72, September 2017, Pages 38-47.
DOI: 10.1016/j.oraloncology.2017.07.003.
Authors: César Rivera, Ana Karina de Oliveira, Rute Alves Pereira e Costa, Tatiane De Rossi & Adriana Franco Paes Leme.

2.2 Agrin is a hub in oral cancer. Submitted to The Journal of Oral Pathology (Annex 4).
Authors: César Rivera, Flávia S Zandonadi, Celeste Sánchez-Romero, Ciro D Soares, Wilfredo A González-Arriagada & Adriana Franco Paes Leme.

2.1 Prognostic biomarkers in oral squamous cell carcinoma: a systematic review

Running title: Biomarkers for oral cancer

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Conflict of interest statement: The authors had no conflict of interest concerning the topic under consideration in this article.

ABSTRACT

Over the years, several tumor biomarkers have been suggested to foresee the prognosis of oral squamous cell carcinoma (OSCC) patients. Here, we present a systematic review to identify, evaluate and summarize the evidence for OSCC reported markers. Eligible studies were identified through a literature search of MEDLINE/PubMed until January 2016. We included primary articles reporting overall survival, disease-free survival and cause-specific survival as outcomes. Our findings were analysed using REporting recommendations for tumor MARKer prognostic studies (REMARK), QuickGo tool and SciCurve trends. We found 41 biomarkers, mostly proteins evaluated by immunohistochemistry. The selected studies are of good quality, although, any study referred to a sample size determination. Considering the lack of follow-up studies, the molecules are still potential biomarkers. Further research is required to validate these biomarkers in well-designed clinical cohort-based studies.

Keywords. mouth neoplasms; oral cancer; oral squamous cell carcinoma; biomarkers, tumor; review, systematic

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck (excluding nonmelanoma skin cancer), with more than 300,000 new cases reported annually worldwide[1]. The disease has a high morbidity rate (37.8%) five years after diagnosis (http://www.cancer.gov/statistics/find - 2003-2009 data); despite the progress in research and therapy, survival has not improved significantly in the last few decades [2]. The search for prognostic markers represents a continuing challenge for biomedical science.

A cancer biomarker may be a molecule secreted by a tumor cell or a specific response of the body to the presence of cancer [3]. Biomarkers can be used for patient assessment in multiple clinical settings, including estimating the risk of disease and distinguishing benign from malignant tissues [4]. Cancer biomarkers can be classified based on the disease state, including predictive, diagnosis and prognosis biomarkers [5]. A prognostic biomarker informs about a likely cancer outcome (e.g., overall survival, disease-free survival, and cause-specific survival) independent of treatment received [6].

According to the NCI Dictionary of Cancer Terms (https://www.cancer.gov/publications/dictionaries/cancer-terms) the overall survival (OS) corresponds to the length of time from either the date of diagnosis or the start of treatment for cancer, which patients diagnosed with the disease are still alive. Disease-free survival (DFS, also called relapse-free survival) offers the length of time after primary treatment ends that the patient survives without any signs or symptoms of that cancer. Cause-specific survival (CSS) is the length of time from either the date of diagnosis or the start of treatment for cancer to the date of death from the disease.

From the identification of a promising biomarker to its clinical use, there is a long pathway involving many complicated hurdles, such as estimating the number of patients needed for the validation phase and statistical validation, among others [7, 8]. This validation and qualification are responsible for linking the promising biomarker with a biological process to clinical endpoints [9].

Considering several tumor biomarkers have been suggested to predict the prognosis of OSCC patients, we performed a systematic review, which is widely accepted as a ”gold standard” in medicine based on evidence [10], to identify, evaluate and summarize the evidence for OSCC reported markers.

METHODS

We performed a systematic review to conduct this investigation. The independent variables were prognostic biomarkers; the dependent variables were OSCC outcomes.

Search strategy. A systematic review allows critical analysis of multiple research studies. Aiming to answer the question "what are the biomarkers of OSCC?", a systematic literature search based on keywords was performed. As PubMed comprises more than 26 million citations from the biomedical literature from MEDLINE, it is the search engine of choice to initiate queries in the health sciences. To identify all the primary research studies that evaluated candidate biomarkers in OSCC, we searched the MEDLINE/PubMed (http://www.ncbi.nlm.nih.gov/pubmed) medical literature database up to January 18, 2016. The search strategy was based on combinations of the following keywords: "mouth neoplasms" [MeSH] and "biomarkers" [MeSH] and (risk ratio [Title/Abstract] or relative risk [Title/Abstract] or odds ratio [Title/Abstract] or risk [Title/Abstract]) and ("humans"[MeSH Terms] and English [lang]).

Inclusion criteria. Articles were included based on a previously published protocol [11]. Briefly, studies were selected if they examined the impact of a potential biological marker on at least one of the features in OSCC patients: OS, DFS or CSS. These definitions were assessed among the selected papers. In addition, if a study was focused on isolated or combined (multiple) tumor biomarkers, it must have been subjected to multivariable analysis with one or more additional variables.

Exclusion criteria. Articles were excluded from the present review for the following reasons: i) lack of the terms "oral cancer" and "risk" in their titles, abstracts or keywords; ii) absence of risk ratios and iii) unclear defining criteria for groups and variables.

Potential prognostic biomarker. To determine whether a biomarker is potentially prognostic, the selected articles showed: i) a formal test (binary logistic regression or Cox proportional hazards model) and ii) a statistically significantly association between the biomarker and outcome [6]. The computed risk (odds ratio, OR or hazard ratio, HR) was reported as the risk of a specific outcome from the biomarker group versus the reference group, with OR/HR>1 indicating increased risk and OR/HR<1 indicating decreased risk.

Data extraction. One investigator reviewed all the eligible studies and carefully extracted the study characteristics, including the article citation information, biomarker name and classification, condition or outcome, laboratory technique, sample size, number of clinical outcomes, status of biomarker expression, statistical test method, computed risk and its p-value and 95% confidence interval (CI). The main biological processes in which the biomarkers are involved were obtained using QuickGo (http://www.ebi.ac.uk/QuickGO).

Quality assessment. Quality assessment was performed in duplicate for each eligible study by three independent reviewers using operationalized prognostic biomarker reporting the REMARK guidelines [12] and extracted details on 20 items. The inter-observer agreement was evaluated using Kappa statistics.

Publication trends. To observe the publication trends in the selected potential OSCC biomarkers, we searched the scholarly literature in SciCurve Open (http://www.scicurve.com). SciCurve Open is a search engine that transforms a systematic literature review into an interactive and comprehensible environment [13].

RESULTS

Studies searching for OSCC biomarkers: proteins are the most analysed molecules. The keyword search strategy identified 403 suitable abstracts, from which 320 were excluded by reviewing the title and abstract during the screen because they did not meet the eligibility criteria. Full text articles were obtained for 83 studies (34 with single markers and 49 with multiple or combined markers).

Forty-five of these articles were excluded for different reasons, including: out of goal (3 articles), unavailability online (2 articles), lack of multivariable analysis (18 articles) and model inconsistencies (22 articles). Figure 1 shows a PRISMA diagram for this review (for details, see Supplemental file S1, http://ars.els-cdn.com/content/image/1-s2.0-S1368837517301938-mmc1.xlsx).

The selected studies were screened, and specific study characteristics and remarks were recorded. These parameters are summarized in Table 1 (the article context is grouped according to the hallmarks of cancer [14]). Thirty-eight papers examined 41 biomarkers [15-52]. Most of them were proteins determined using immunohistochemistry (IHC) in paraffin-embedded tissues (36 of 38 studies).

The included studies were conducted in Poland, India, Germany, Taiwan, Korea, Japan, Australia, Spain, China, Portugal, Brazil, UK, USA and Finland. Variable cohort sizes were used, ranging from 34 to 208 patients. *n*, outcome event number, statistical test, CIs and p-values, risk values and Google scholar citations were extracted (see Supplemental file S1). The main results of the included articles are summarized in Table 2. The biomarker high *vs*. low levels was defined differently in each study.

Fourteen clinicopathologic group factors were incorporated in 48 multivariate analyses (38 studies generated 48 significant models and 210 covariables). The most commonly included prognostic factors for model adjustment were the histopathological features (excluding the WHO histological differentiation degree) in 30 models (62,5%), protein (27 models, 53,3%) AJCC clinical stage (22 models, 45,8%) and WHO histological differentiation degree (21 models, 43,8%) (Figure 2). For complete details, see Supplemental file S1.

Quality of study reports: studies do not clear determine the sample size. The result of this agreement was 0.87, which is classified as almost perfect. Differences were resolved by consensus. Most study analyses reported details of the objective/hypothesis, patient source, population characteristics, assay method, cut-off point, and relationship of the potential marker to standard prognostic variables, as well as discussed the implications for future research and clinical value (for details, see Supplemental file S2, http://ars.els-cdn.com/content/image/1-s2.0-S1368837517301938-mmc2.xlsx). Notably, no study referred to a statistical sample size, which is key for biomarker validation.

Proposed OSCC biomarkers. None of the studied molecules presented an analysis of validation, so we called them "potential biomarkers". A narrative review of the proposed biomarkers is presented in Table 3.

Trends: potential biomarkers with more publications and citations. To explore the publication trends in our OSCC potential protein biomarkers, we searched the scholarly literature in SciCurve Open. SciCurve uses PubMed's library of 23 million references to generate visually pleasing graphs and curves that help grasp trends in the literature [53]. It is associated with the following main functionalities: publications, citations, most prolific authors and countries.

According to Figure 3, MMP-2 is the most researched field, followed by MMP-1, cadherin-1 and mucin-1. The countries with the largest contributions are the USA, Japan and China.

DISCUSSION

We have summarized the results on the association between biomarkers and oral cancer outcomes using a systematic review. Overall, our results suggest 41 prognostic molecules involved with OSCC endpoints. These markers may be candidates for long-term studies.

OSCC is the most relevant epithelial malignancy for dental surgeons. It has late clinical detection and poor prognosis, and the available therapeutic alternatives are highly expensive and disfiguring [54].

OSCC is a very complex subtype of cancer with high heterogeneity [55]. Several risk factors are implicated in its aetiology, among which tobacco, alcohol, viruses and diet are highlighted [2]. These factors related to genetic inheritance may have a carcinogenic effect on the normal cells of the respiratory and digestive systems. This type of carcinoma can occur anywhere in the mouth, although the most affected sites are the tongue, lower lip and mouth floor [2, 56]. These regions are great facilitators of carcinoma spreading to regional lymph nodes and/or distant organs [57]. At present, the diagnosis of OSCC is based on comprehensive clinical examination and histological analysis of suspicious areas [58]. Recently, The Cancer Genome Atlas (TCGA) showed that a large dataset of proteomics/genomics did not improve the prognosis potential of classic clinical variables in patients with different types of cancer [59]. Some studies seeking biomarkers in oral cancer are still in the discovery phase, requiring validation to be accepted in clinical practice.

Currently, biomarkers are a subject of particular interest because they may represent the most important part in the diagnosis step. In the future, specific and personalised diagnostics can guide treatment against the disease and consequently improve the chance of curing the disease.

In response to the need for tumor biomarkers for OSCC that can be readily evaluated in routine clinical practice, we performed a systematic review (PubMed *keyword-base* query) of the published literature to identify single or multiple biomarkers for OSCC outcomes: overall survival, disease-free survival, relapse-free survival and cause-specific survival. The main finding was the identification of 38 studies describing multivariate survival analysis for 41

biomarkers. From these articles, MMP-2, MMP-1, cadherin-1, mucin-1, GLUT-1 (SLC2A1), mucin-4, interleukin-8, HPV-16, EGFR and p53 have received great interest from the scientific community. Of these, up to now, it is accepted that the HPV status have a clinical utility [60], suggesting that HPV positive head and neck squamous cell carcinomas form a distinct clinical entity with better treatment outcome [61].

The malignant progression to OSCC is characterized by the acquisition of progressive and uncontrolled growth of tumor cells. Predicting whether premalignant lesions will progress to cancer is crucial to make appropriate treatment decisions. The first detectable clinical changes that can indicate that an epithelium is on the way to establish OSCC is the occurrence of malignant disorders, including leukoplakia (most common) [2]. In this context, we emphasize the results associated with Rho GTPase-activating protein 7, retinal dehydrogenase 1/prominin-1 (combined biomarkers), podoplanin, cortactin/focal adhesion kinase 1 (combined biomarkers) and catenin delta-1. These proteins show a potential role as a marker of oral cancer risk and malignant transformation [17, 26-28, 39, 40, 42].

There are thousands of papers reporting cancer biomarker discovery, but only few clinically useful biomarkers have been successfully validated for routine clinical practice [62]. Quality assessment tools have been developed for prognostic studies to help identify study biases and causes of heterogeneity when performing meta-analysis. We chose to use the REMARK reporting guidelines, which provide a useful start for assessing tumor prognostic biomarkers (all included studies were prognostic). We found that the investigations reported an average of 19 of 20 REMARK items. However, all studies failed to report the sample size calculation. In the absence of this calculation, the findings of each research should be interpreted with caution [63]. The sample size requirements that allow the identification of a benefit beyond existing biomarkers are even more demanding [64].

In our review, none of the articles that created prediction models had internal or external validation. In general, studies recruited cases of OSCC from a clinical setting as well as controls without a clearly defined diagnosis. Under this circumstance, any differences in the biomarker levels between OSCC patients and controls could simply reflect individual differences rather than cancer-related differences. The lack of biomarker validation strategies and standard operating procedures for sample selection in the included studies represent an important pitfalls and limitations, leading us to use the term "potential biomarkers".

It is important to highlight that our research searched only one database, which means that only studies available in MEDLINE were included. Additionally, due to the heterogeneity among the studies, a meta-analysis that combined the results of different studies could not be

performed. In addition, our research included results from observational studies, and their evaluation may have been problematic if the *confounder variables* were *not* adjusted because *they* were *not* measured [65].

CONCLUSION

Recent research in OSCC has identified a multitude of potential markers that have a significant role in prognosis. In this systematic review, despite the inherent limitations, we identified several potential biomarkers of particular interest that appear to carry prognostic significance. Considering the validation step as a process of assessing the biomarker and its measurement performance characteristics, and determine the range of conditions under which this biomarker can provide reproducible data [9], our results show biomarkers in the discovery phase, thereby leading us to call them OSCC "potential biomarkers". Nevertheless, it is urgent to apply validation methods to provide clinically useful oral cancer biomarkers.

ACKNOWLEDGMENTS

CONICYT Becas-Chile Scholarship 8540/2014, PNPD/CAPES 33003033009P4, and FAPESP Grants 2016/07846-0, 2014/06485-9 and 2015/12431-1, supported this work.

FIGURE CAPTIONS

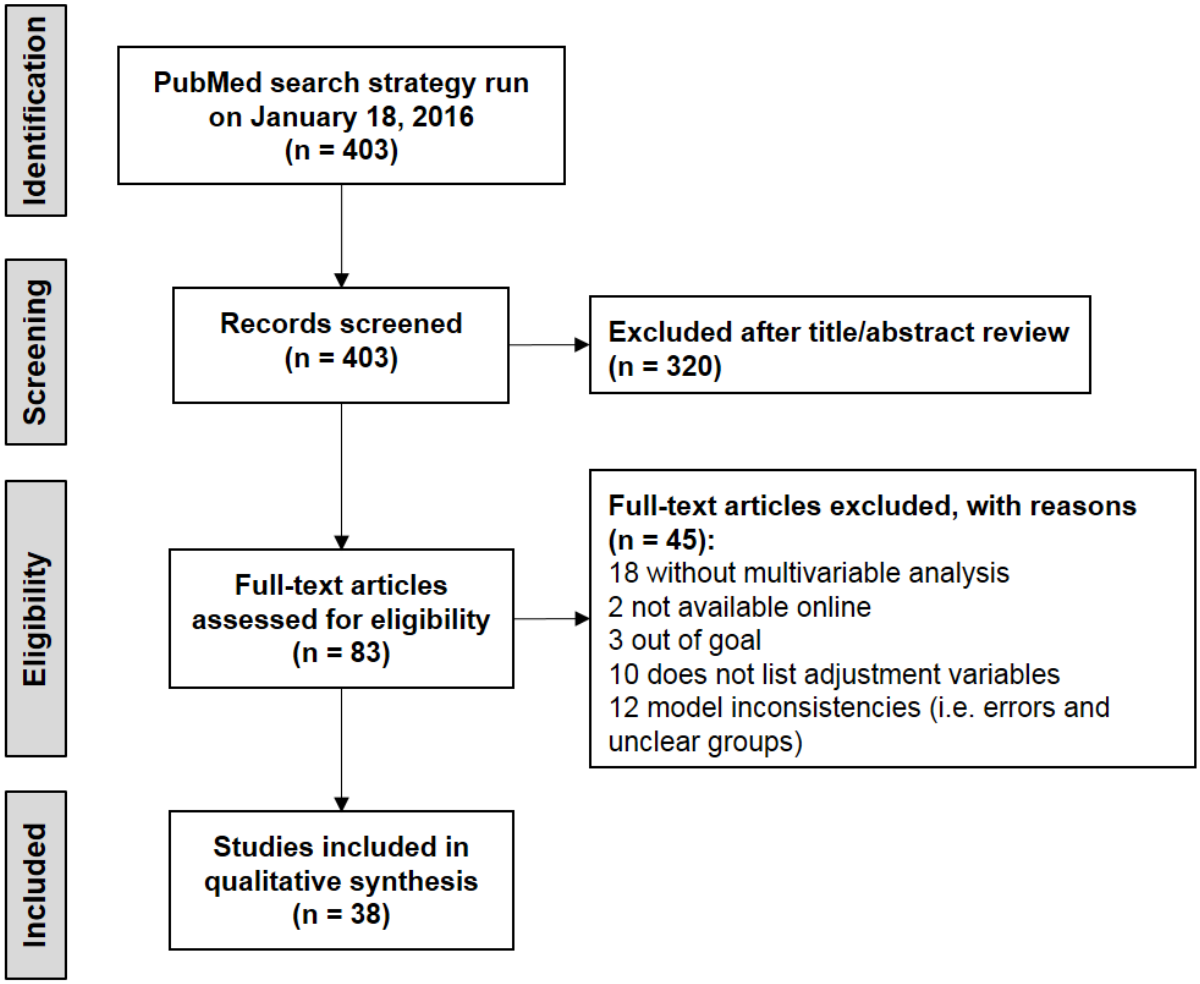

Figure 1. Flow diagram representing systematic literature search on biomarkers and oral cancer outcomes. Studies were included if they examined the impact of a potential biomarker on at least one of overall survival, disease free survival or cause-specific survival in oral squamous cell carcinoma patients.

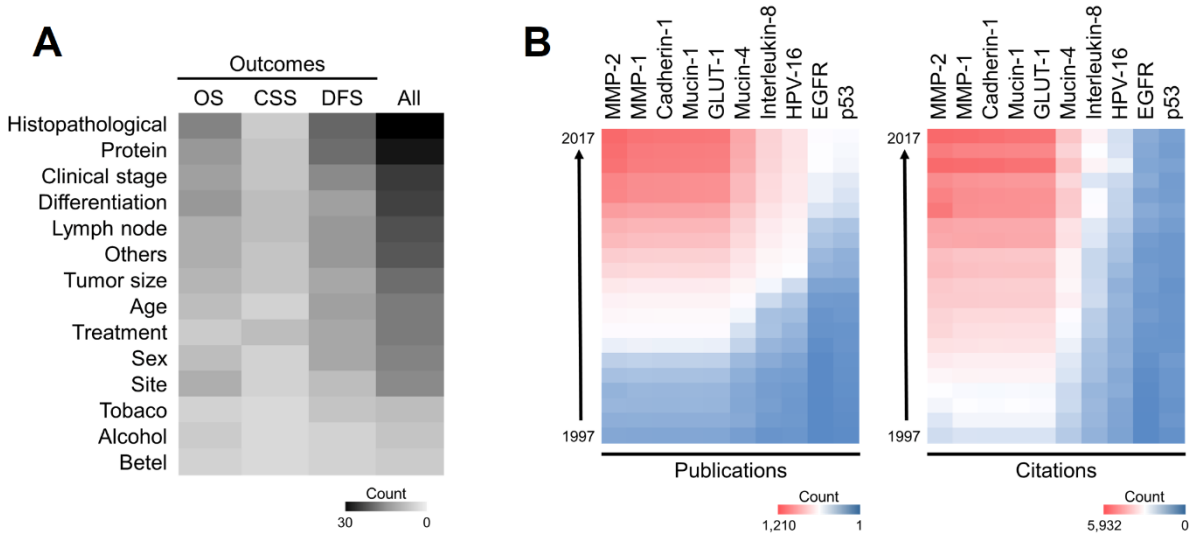

Figure 2. (A) Adjustment variables. Frequencies with which adjustments were performed for OSCC outcomes. The heat map combines the most frequent factors for adjustments and survival models. The most commonly included factor was "histopathological features" (excluding the WHO histological differentiation degree). Higher numbers represent intense and saturated colors. (**B)** Trends in oral cancer biomarkers (top ten). Compared with other biomarkers, MMP-2 is the most researched field with 15,057 publications and 46,368 citations (1997-2017). MM-2 is followed by MMP-1 (14,650 publications/43,762 citations) and cadherin-1 (14,531/43,422).

Table 1. Characteristics of the included studies.

Reference	Biomarker*	Change	Design and method	Study remarks
Sustaining proliferative signaling				
Gontarz M et al., 2014	Proliferation marker protein Ki-67 or ki-67 (MKI67)	(+)	Poland. Retrospective. IHC.	May be useful in the selection of patients at a higher risk of recurrence who would benefit from postoperative radiotherapy
Ramshankar V et al. 2014	Cyclin-dependent kinase inhibitor 2A or p16 (CDKN2A)	(+)	India. Retrospective. IHC and RT-qPCR.	CDKN2A overexpression is a single important prognostic variable in defining a high risk group. CDKN2A expression should possibly not be used as a surrogate marker for HPV infection in tongue cancers.
	Human papillomavirus type 16 or HPV-16 (HPV16)	(+)		
Tripathi SC et al., 2012	Rho GTPase-activating protein 7 or DLC1 (DLC1)	(-)	India. Retrospective. IHC.	Loss of expression emerged as an important biomarker for predicting patients diagnosed with oral dysplasia at high risk of transformation. Is a poor prognostic marker for oral squamous cell carcinoma patients.
Freudlsperger C et al., 2011	MKI67	(+)	Germany. Retrospective. IHC.	Expression level could be used to identify a subgroup of surgically treated patients with stage I OSCC who might benefit from treatment intensification.
Kok SH et al., 2010	Protein CYR61 (CYR61)	(+)	Taiwan. Retrospective. IHC.	Is a positive growth modulator of OSCC and overexpression is an independent prognostic indicator.
Shah NG et al. 2009	Cellular tumor antigen p53 or p53 (TP53)	(+)	India. Retrospective. IHC.	TP53 was independently associated with DFS and OS, and CDKN2A with DFS only.
	CDKN2A	(-)		
Kim SJ et al. 2007	Carbonic anhydrase 9 (CA9) and MKI67 combined	(+)	Korea. Retrospective. IHC.	The expression of CA9 and MKI67may be useful for predicting prognosis in squamous cell carcinoma of the tongue.

Shiraki M et al. 2005	TP53, G1/S-specific cyclin-D1 (CCND1), epidermal growth factor receptor (EGFR) combined	(+)	Japan. Retrospective. IHC.	Simultaneous expression of these markers in oral cancers might prove to be a useful indicator for identification of low- or high-risk patients.
Myo K et al., 2005	CCND1	(+)	Japan. Retrospective. FISH.	Aberrations in gene numbers appear to be valuable in identifying patients at high risk of late lymph node metastasis in stage I and II OSCCs.
Pande P et al. 2002	TP53	(+)	India. Prospective. IHC.	RB1 loss and TP53 overexpression may serve as adverse prognosticators for disease free survival of the patients.
	Retinoblastoma-associated protein (RB1)	(-)		
Bova RJ et al. 1999	CCND1	(+)	Australia. Retrospective. IHC.	CCND1overexpression and loss of CDKN2A expression predict early relapse and reduced survival in squamous cell carcinoma of the anterior tongue
	CDKN2A	(-)		
Evading growth suppressors				
Pérez-Sayáns M et al., 2014	Myc proto-oncogene protein (MYC)	(+)	Spain. Retrospective. IHC.	Its determination can be valuable when used together with other markers to assess the prognosis of OSCC patients.
Liu W et al. 2013	Retinal dehydrogenase 1 or ALDH1 (ALDH1A1)	(+)	China. Prospective. IHC.	Expression of cancer stem cell markers ALDH1A1 and PROM1 correlate with a high risk of malignant transformation in a large series of patients with premalignant oral leukoplakia.
	Prominin-1 or CD133 (PROM1)	(+)		
Feng JQ et al. 2013	ALDH1A1	(+)	China. Retrospective. IHC.	Expression pattern was associated with malignant transformation, suggesting that it may be valuable predictors for evaluating the risk of oral cancer.
Suzuki F et al., 2005	Protein S100-A2 (S100-A2)	(-)	Japan. Retrospective. IHC.	Patients with stage I or II invasive OSCC without expression should be considered a high-risk group for late cervical metastasis when a wait-and-see policy

				for the neck is being considered.
Tsai ST et al., 2005	S100-A2	(-)	Taiwan. Retrospective. IHC.	Loss of nuclear expression may serve as an independent prognostic marker for early-stage oral cancer patients at high risk of recurrence. A more aggressive treatment modality and intensive follow-up may be recommended for the patients with reduced expression of in tumor cell nuclei.
Resisting cell death				
Moura IM et al., 2014	Cell division cycle protein 20 homolog (CDC20)	(+)	Portugal. Retrospective. IHC.	High expression is associated with poor prognosis in OSCC, may be used to identify high-risk OSCC patients, and may serve as a therapeutic target.
Tang JY et al., 2013	Microtubule-associated proteins 1A/1B light chain 3A or LC3 (MAP1LC3A)	(+)	Taiwan. Retrospective. IHC.	Elevated expression, which corresponds to increased level of autophagy activity, is a frequent event and an indicator of poor prognosis in human OSCC.
de Carvalho-Neto PB et al. 2013	Tumor necrosis factor receptor superfamily member 6 or FAS (FAS)	(-)	Brazil. Retrospective. IHC.	DFS and CSS were significantly correlated with FAS/FASL expression profiles. The high risk category was an independent marker for earlier disease relapse and disease-specific death.
	Tumor necrosis factor ligand superfamily member 6 or FASL (FASLG)	(-)		
Inducing angiogenesis				
Yanagawa T et al., 2004	Heme oxygenase 1 (HMOX1)	(-)	Japan. Retrospective. IHC.	Could be used clinically as a marker for tumors possessing the potential for lymph node metastasis. This method could prove useful as an adjuvant method to detect lymph node metastasis and may help reduce the number of

				surgeries by indicating when surgery is unnecessary.
Activating invasion and metastasis				
de Vicente JC et al., 2013	Podoplanin (PDPN)	(+)	Spain. Retrospective. IHC.	Could be a valuable biomarker for risk assessment of malignant transformation in patients with oral leukoplakia along with histological assessment
de Vicente JC et al. 2012	Src substrate cortactin (CTTN) and focal adhesion kinase 1 (PTK2) combined	(+)	Spain. Retrospective. IHC.	Strong immunoexpression of CTTN and PTK2, and not only one of them, is a predicting factor for increased cancer risk in oral premalignant lesions.
Hamada T et al., 2012	Mucin-4 (MUC4)	(+)	Japan. Retrospective. IHC.	Overexpression is an independent factor for poor prognosis of patients with OSCC; therefore, patients with OSCC showing positive expression should be followed up carefully.
Ma LW et al., 2012	Catenin delta-1 (CTNND1)	(+)	China. Retrospective. IHC.	May serve as a useful marker for the identification of a high risk of potentially malignant oral lesions progressing to OSCC
Marsh D et al. 2011	Actin, aortic smooth muscle or SMA (ACTA2)	(+)	UK. Retrospective. IHC.	An positive, myofibroblastic stroma is the strongest predictor of OSCC mortality.
Zhang Z et al. 2011	Interstitial collagenase or MMP-1 (MMP1)	(+)	China. Retrospective. IHC.	Up-regulation of MMP1, MMP2 might be important features of OSCC progression.
	72 kDa type IV collagenase or MMP-2 (MMP2)	(+)		
Liu LK et al. 2010	Vimentin (VIM)	(+)	China. Retrospective. IHC.	The high expression of VIM and low expression of CDH1were associated with survival and were independent prognostic factors in multivariate analyses.
	Cadherin-1 (CDH1)	(-)		
Kawaguchi H et al., 2008	PDPN	(+)	USA. Retrospective. IHC.	Together with histology, may serve as a powerful biomarker to predict the risk for oral cancer development in patients with oral leukoplakia.

Pukkila M et al., 2007	VCAN protein (VCAN)	(+)	Finland. Retrospective. IHC.	Correlated with both increased risk for disease recurrence and shortened survival. High stromal expression may thus be considered an independent and adverse prognostic marker in OSCC.
Endo K et al. 2006	E3 ubiquitin-protein ligase AMFR (AMFR)	(+)	Japan. Retrospective. IHC.	Is valuable in identifying patients at high risk for tongue SCC recurrences
Reprogramming of energy metabolism				
Hamada T et al., 2012	Mucin-1 (MUC1)	(+)	Japan. Retrospective. IHC.	Is a risk factor for subsequent lymph node metastasis in patients with OSCC and therefore may represent an indication for elective neck dissection
Eckert AW et al. 2011	Hypoxia-inducible factor 1-alpha (HIF1A) or HIF-1α and Solute carrier family 2, facilitated glucose transporter member 1 or GLUT-1 (SLC2A1) combined	(+)	Germany. Retrospective. IHC.	Coexpression of high levels of HIF1A and SLC2A1 is significantly correlated with prognosis in OSCC patients.
Eckert AW et al., 2010	HIF1A	(+)	Germany. Retrospective. IHC.	Immunohistochemical detection appears to improve diagnosis and to provide prognostic information in addition to the TNM – system and histological grade of OSCC.
Fillies T et al., 2005	HIF1A	(-)	Germany. Retrospective. IHC.	Overexpression is an indicator of favorable prognosis in T1 and T2 SCC of the oral floor. Node negative patients lacking expression may therefore be considered for adjuvant radiotherapy.
Tumor-promoting inflammation				
Kwon M et al., 2015	Interleukin-4 receptor subunit alpha	(+)	Korea. Retrospective. IHC.	High expression of IL4R correlated with increased recurrence, while high

	(IL4R)			IL13RA1 expression had an inverse relationship to recurrence and disease-specific survival in OSCC patients.
	Interleukin-13 receptor subunit alpha-1 (IL13RA1)	(+)		
Fujita Y et al. 2014	Interleukin-8 (CXCL8)	(+)	Japan. Retrospective. IHC.	These factors in addition to N status may have prognostic value in patients with resectable OSCC.
	Scavenger receptor cysteine-rich type 1 protein M130 (CD163)	(+)		
Lai WM et al. 2013	Myeloperoxidase (MPO)	(+)	Taiwan. Retrospective. IHC.	Higher MPO expression in buccal mucosal SCC is a risk factor for second primary tumors.
Huang SF et al. 2012	Serpin B3 (SERPINB3) and C-reactive protein (CRP) combined	(+)	Taiwan. Retrospective. Immunoassay.	High levels of both preoperative SERPINB3 and CRP levels act as a predictor for DFS and OS.

The articles are grouped according to the hallmarks of cancer. *UniProt Knowledgebase or common name. HGNC name between parentheses. (+) Up-regulated/overexpressed, (-) Down-regulated/down-expressed, CSS, cause-specific survival; OS, overall survival; DFS, disease free survival; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

Table 2. Data extracted from selected studies.

Article	Biomarker*	N**	Cases vs. reference group	Outcome	HR	CI	p-value
Sustaining proliferative signaling							
Gontarz M et al., 2014	MKI67	34	IHC. High vs. low.	DFS CSS	5.42 9.02	1.18–24.83 1.99–40.93	0.029 0.004
Ramshankar V et al. 2014	CDKN2A	156	IHC. Overexpression vs. low	OS DFS	2.34 2.58	1.30-4.40 1.44-4.64	0.005 0.002
	HPV16		RT-qPCR. Negative vs. positive	OS	0.61	0.38-0.99	0.049
Tripathi SC et al., 2012	DCL1	181	IHC. Loss vs. expression	DFS	2.10	1.2-3.9	0.023
Freudlsperger C et al., 2011	MKI67	69	IHC. High vs. low	DFS	4.24	NS.	0.029
Kok SH et al., 2010	CYR61	93	IHC. High vs. low	OS	2.44	1.20–4.95	0.010
Shah NG et al. 2009	TP53	135	IHC. Positive vs. negative	OS DFS	2.71 2.45	1.29-5.72 1.28-4.69	0.009 0.007
	CDKN2A		IHC. Negative vs. positive	DFS	2.08	1.05-4.14	0.036
Kim SJ et al. 2007	CA9 and MKI67 combined	60	IHC. High vs. low	OS DFS	4.04 2.39	NS NS	0.005 0.007
Shiraki M et al. 2005	TP53, CCND1 and EGFR combined	140	IHC. Co-expression of all three markers vs. 0-2 markers	OS	3.56	1.59–7.93	0.002
Myo K et al., 2005	CCND1	45	Numerical aberration positive vs. negative	DFS	8.69	2.23–33.80	0.002
Pande P et al. 2002	TP53	50	IHC. Positive vs. negative	DFS	2.98	1.04-8.47	0.041
	RB1		IHC. Negative vs. positive	DFS	2.94	0.12-0.92	0.034
Bova RJ et al. 1999	CCND1	147	IHC. Positive vs. negative	DFS	2.48	1.0-6.15	0.005
	CDKN2A	143	IHC. Negative vs. positive	OS	3.15	1.65-7.50	0.001
Evading growth suppressors							

Pérez-Sayáns M et al., 2014	MYC	NA	NA	OS	1.15	1.06-1.25	<0.001
Liu W et al. 2013	ALDH1A1	141	IHC. Positive vs. negative	DFS	4.17	1.96–8.90	<0.001
	PROM1		IHC. Positive vs. negative	DFS	2.86	1.48–5.55	0.002
Feng JQ et al. 2013	ALDH1A1	34	IHC. Positive vs. negative	DFS	8.89***	1.67–47.41	0.011
Suzuki F et al., 2005	S100-A2	52	IHC. Negative vs. positive	DFS	0.20	0.08-0.53	0.001
Tsai ST et al., 2005	S100-A2	70	IHC. Low vs. high	DFS	4.36	1.52-12.49	0.006
Resisting cell death							
Moura IM et al., 2014	CDC20	65	IHC. Positive vs. negative	CSS	2.36	1.08–5.17	0.032
Tang JY et al., 2013	MAP1LC3A	90	High vs. low	OS	2.99	1.39-7.05	0.004
de Carvalho-Neto PB et al. 2013	FAS	60	IHC. Negative vs. positive	DFS	3.73	1.16-11.95	0.027
	FASLG		IHC. Negative vs. positive	CSS	2.58	1.03-6.46	0.044
Inducing angiogenesis							
Yanagawa T et al., 2004	HMOX1	54	IHC. Low vs. high	DFS	8.49***	1.64-44.09	0.010
Activating invasion and metastasis							
de Vicente JC et al., 2013	PDPN	58	IHC. Score 2–3 vs. 0–1	DFS	8.74	1.83-41.63	0.007
de Vicente JC et al. 2012	CTTN and PTK combined	50	IHC. High co-expression of vs. negative to moderate	DFS	6.30	1.55-25.58	0.01
Hamada T et al., 2012	MUC4	150	IHC. Positive vs. negative	OS	1.62	1.12-2.41	0.002
Ma LW et al., 2012	CTNND1	68	Phosphorylated. IHC. High vs. low	DFS	3.43	1.40-8.41	0.007
Marsh D et al. 2011	ACTA2	208	IHC. High vs. low	OS	3.06	1.65-5.66	0.002
Zhang Z et al. 2011	MMP1	NS.	IHC intensity in cancer tissue (continuous variable)	DFS	1.09***	1.03-1.16	0.003
	MMP2		IHC intensity in cancer tissue (continuous variable)	DFS	1.03***	1.00-1.05	0.025

Liu LK et al. 2010	VIM	83	IHC. High vs. negative/low	OS	1.61	1.02-2.55	0.042
	CDH1		IHC. Negative/low vs. high	OS	0.58	0.37-0.90	0.016
Kawaguchi H et al., 2008	PDPN	150	IHC. Oral leukoplakia positive vs. negative	DFS	3.09	1.53-6.23	0.002
Pukkila M et al., 2007	VCAN	139	IHC. High vs. low	CSS	1.80	1.01-3.30	0.048
Endo K et al. 2006	AMFR	99	IHC. Positive vs. negative	DFS	2.07	1.04-4.11	0.038
Reprogramming of energy metabolism							
Hamada T et al., 2012	MUC1	206	IHC. Positive vs. negative	OS DFS[1] DFS[2]	2.09 1.71 2.29	1.04-4.29 1.02-2.85 1.08-4.93	0.040 0.040 0.030
Eckert AW et al. 2011	HIF1A and SLC2A1 combined	55	IHC. High co-expression vs. low	CSS	5.13	1.33–19.79	0.017
Eckert AW et al., 2010	HIF1A	80	IHC. Moderate or strong vs. negative or weak	CSS	3.49	NS	0.016
Fillies T et al., 2005	HIF1A	85	IHC. Low vs. very high	OS DFS	0.20 0.30	0.10–0.50 0.10–0.70	0.000 0.010
Tumor-promoting inflammation							
Kwon M et al., 2015	IL4R	186	IHC. High vs. low	DFS	2.34	1.38-3.97	0.002
	IL13RA1		IHC. High vs. low	OS DFS	0.26 0.33	0.14-0.48 0.17-0.67	<0.001 0.002
Fujita Y et al. 2014	CXCL8	50	IHC. Positive vs. negative	DFS	0.27	0.08-0.89	0.031
	CD163		IHC. Invasive front, high vs. low	DFS	2.63	1.31-5.25	0.006
Lai WM et al. 2013	MPO	173	IHC. High vs. low	DFS	3.89	1.33-11.39	0.013
Huang SF et al. 2012	SERPINB3 and CRP combined	99	Immunoassay. Positive vs. negative	DFS OS	8.43 6.25	3.94-18.01 2.60-15.01	<0.001 <0.001

The articles are grouped according to the hallmarks of cancer. *HGNC database recommended names were used. **N, number of subjects in the contrast. ***Odds ratio (multiple logistic regression). HR and OR values are reported as they originally appear in the selected articles. NS, not specified. NA, not apply. DFS, disease free survival; CSS, cause-specific survival; OS, overall survival;. [1]Recurrence and [2]lymph node metastasis.

Table 3. Overview of proposed biomarkers

Name*	**Biological processes**	**Cancer context**
MKI67	Cell cycle, cell proliferation.	Marker of the growth fraction for a certain cell population [66]. The labelling index is considered one of the best prognostic factors of the survival rate and recurrence [56].
CDKN2A	Cell cycle, cell cycle arrest.	This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene [67].
HPV16	High-risk HPV type.	Is emerging as an important factor in the rise of oropharyngeal tumors affecting non-smokers in developed countries. Patients with HPV(+) tumors demonstrated favorable outcomes compared to TP53 mutants and 11q13/*CCND1*-amplified tumors [60].
DLC1	Negative regulation of cell proliferation and migration.	Acts as a tumour suppressor in a number of common cancers, including liver cancer [68].
CYR61	Regulation of cell growth and adhesion.	Can function as an oncogene or a tumour suppressor, depending on the origin of the cancer [69].
TP53	Cell cycle, cell cycle arrest.	Tumor-suppressor protein. Mutations in this gene are associated with a variety of human cancers [67].
CA9	Response to hypoxia.	Is the most widely expressed gene in response to hypoxia. Its role in intracellular pH maintenance represents the means by which cancer cells adapt to the toxic conditions of the extracellular environtment [70]
CCND1	Cell cycle, cell division.	Is frequently deregulated in cancer and is a biomarker of cancer phenotype and disease progression [71].
EGFR	Positive regulation of cell proliferation.	EGFR overexpression is a significant finding in cancer, particularly in head and neck cancer, where it is also associated with a poor prognosis [72].
RB1	Cell cycle, cell cycle arrest.	Tumor-suppressor protein. Defects in this gene are a cause of childhood cancer retinoblastoma (RB), bladder cancer, and osteogenic sarcoma [67].
MYC	Positive regulation of cell proliferation.	Its oncogenic reputation stems from its frequent deregulation in a host of human cancers and from a suite of activities that place this protein at the nexus of cell growth, proliferation, metabolism, and genome stability [73].
ALDH1A1	Ethanol oxidation.	Play a key role in the regulation of growth and differentiation of both normal tissue stem cells and cancer stem cells [74].
PROM1	Retina layer formation.	Maintaining stem cell properties by suppressing differentiation [67].
S100-A2	Endothelial cell migration.	In epithelial tissue, S100-A2 expression is decreased remarkably in tumours compared with normal

		specimens [75]. S100-A2 promotes p53 transcriptional activity, and its loss of expression has been associated with a poorer prognosis and shorter survival [76].
CDC20	Cell cycle, positive regulation of cell proliferation.	The role of CDC20 expression in tumours is not known, but many studies have reported that CDC20 regulates apoptosis, leading to genetically instability [77]
MAP1LC3A	Autophagy.	Strong positive expression in the peripheral area of pancreatic cancer tissue had a shorter overall and disease-free survival; correlations with tumour size, poor differentiation, blood vessel infiltration and tumour necrosis were noted [78].
FAS	Apoptotic process.	Cancer cells can never lose FAS or FASLG. FAS and/or FASLG expression promotes tumor growth and favors the establishment of tumor metastases [79].
FASLG		
HMOX1	Angiogenesis.	Many human tumours produce HMOX1, and its expression is usually higher in cancer cells than in surrounding healthy tissues [80].
PDPN	Lymphangiogenesis.	Is commonly used in the identification of lymphatic endothelial differentiation in vascular endothelial neoplasms and lymphatic invasion by tumours [81]. Recent evidence have identified podoplanin as a marker of cancer-associated fibroblasts [82].
CTTN	Cell motility and focal adhesion assembly.	Is overexpressed in breast cancer and squamous cell carcinomas of the head and neck [67].
PTK2	Angiogenesis.	Promotes tumor progression and metastasis through effects on cancer cells, as well as stromal cells of the tumor microenvironment [83]
MUC4	Cell adhesion.	An aberrant expression of MUC4 has been reported in various carcinomas [84].
CTNND1	Cell adhesion.	Evidence is emerging that complete loss, downregulation or mislocalization of CTNND1 correlates with the progression of different types of human tumours [85].
ACTA2	Mesenchyme migration.	Patients with lung adenocarcinomas and high ACTA2 expression showed significantly enhanced distant metastasis and unfavorable prognosis [86].
MMP1	Proteolysis.	Imbalance between matrix metalloproteinases and their inhibitors play the important role in progression of head and neck cancer [87].
MMP2	Angiogenesis, response to hypoxia and proteolysis.	
VIM	Movement of cell or subcellular component.	Has been recognized as a marker for epithelial-mesenchymal transition. Overexpression in cancer correlates well with accelerated tumor growth, invasion, and poor prognosis [88].

CDH1	Cell adhesion.	Loss of function of this gene is thought to contribute to cancer progression by increasing proliferation, invasion, and/or metastasis [67].
VCAN	Cell adhesion.	Is strongly associated with a poor outcome for many different cancers. Depending on the cancer nature, is expressed either by cancer cells themselves or by stromal cells surrounding the tumour [89].
AMFR	Movement of cell or subcellular component.	Is a tumor motility-stimulating protein secreted by tumor cells [67].
MUC1	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	Is aberrantly glycosylated and overexpressed in various epithelial cancers and plays a crucial role in the progression of the disease [90]. MUC1 is often used as a diagnostic marker for metastatic progression [91].
HIF1A	Angiogenesis, response to hypoxia.	Up-regulates the expression of proteins that promote angiogenesis, anaerobic metabolism, and many other survival pathways [92].
SLC2A1	Glucose transport.	Was significantly correlated with depth of invasion and clinical stage in patients with gastric cancer [93].
IL4R	Immune system process and regulation of cell proliferation	The IL4/IL4R signaling axis is a strong promoter of pro-metastatic phenotypes in epithelial cancer cells including enhanced migration, invasion, survival, and proliferation [94].
IL13RA1	Cell surface receptor signaling pathway.	Glioblastoma samples presented higher IL13RA1 and IL13RA2 expression levels compared to lower grades astrocytomas and non-neoplastic cases [95].
CXCL8	Angiogenesis, movement of cell or subcellular component and chemotaxis	Neovascularisation is now recognised as a critical function of CXCL8 in the tumour microenvironment [96].
CD163	Inflammatory response.	Could be used as a general anti-inflammatory myeloid marker with prognostic impact for breast cancer patients [97].
MPO	Defense response.	Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis is an indicator of colorectal cancer risk [98].
SERPINB3	Positive regulation of cell proliferation.	Promotes oncogenesis and epithelial-mesenchymal transition [99]
CRP	Inflammatory response.	Patients with a high baseline CRP had a greater risk of early death compared with those with low CRP levels [100].

*HGNC database recommended names were used. **Representative processes from QuickGo (http://www.ebi.ac.uk/QuickGO).

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2.2 Agrin is a hub in oral cancer progression

Running title: Agrin promotes oral cancer progression

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Conflict of interest statement: The authors had no conflict of interest concerning the topic under consideration in this article.

ABSTRACT

Oncogenic processes that could associate agrin and oral cancer have been identified, but these findings have not been observed in the context of oral cancer progression. Here, we show that silencing agrin interferes with OSCC tumor progression, both *in vitro* and *in vivo*. Agrin silencing suppressed cancer progression-associated events, including cell migration, proliferation, invasion, colony and tumor spheroid formation, and it also decreased the mRNA levels of the OSCC oncogene, *EGFR*. In a mouse orthotopic model, silencing of agrin reduced tumor severity in terms of ulceration, growth pattern and vascular and neural invasion. To gain insights into interaction partners in the agrin complex, we expressed its soluble C-terminus (which has been identified as a potential marker for colorectal cancer), and we identified that its protein network predicts a poor clinical prognosis. Together, our results demonstrate that agrin is important for oral cancer-associated events and is a strong candidate as a therapeutic target for OSCC. This study deepens the understanding of the pathophysiological role of agrin in oral cancer progression.

Keywords. agrin; head and neck neoplasms; mouth neoplasms; oral cancer; biomarkers; tumor; proteomics; protein interaction maps

INTRODUCTION

Worldwide, head and neck squamous cell carcinoma (HNSCC) affects over 500,000 patients per year [1]. Oral squamous cell carcinoma (OSCC) represents 95% of all forms of HNSCC [2]. It is the most common malignancy of the head and neck [3]. Despite advancements in prevention and multimodality therapies, the prognosis of OSCC patients has remained unfavorable in the last few decades [4,5]. A better understanding of the cellular and molecular mechanisms that promote the progression of OSCC can help improve our approach to this disease.

The process of cancer progression (i.e., local invasion and metastasis) is characterized by rapid cellular growth accompanied by alterations of the microenvironment of the cancer cells [6]. The extracellular matrix (ECM) modulates the hallmarks of cancer, and changes in its dynamics contribute to tumor progression [4]. Some components of the ECM, which include heparan sulfate proteoglycans, are frequently overproduced in cancer [7]. Agrin is one of the main heparan sulfate proteoglycans present in the ECM.

Agrin is a multi-domain protein expressed as either a membrane protein or secreted in the ECM [8]. The best characterized function of agrin is in synaptic stabilization during development of the neuromuscular junction [9,10]. Agrin has been shown to act as a sensor in developing oncogenic signals associated with the ECM in hepatic carcinomas [8]. In a previous study, we demonstrated that agrin induces OSCC cell adhesion and migration [11], suggesting that agrin also has an oncogenic role in oral cancer.

Agrin can be proteolytically cleaved, which generates bioactive fragments that modulate cellular behavior [12]. One of the agrin cleavage products, the C-terminal fragment (hereafter called Ct-agrin), has been shown to be a promising new biomarker for pathological processes including sarcopenia [13,14], renal dysfunction [15,16] and colorectal cancer [17]. Within OSCC are proteases, such as MMP-3 and neurotrypsin [18,19], that are capable of generating this soluble fragment.

Despite the findings from the aforementioned studies, the contribution of agrin to OSCC remains unknown. Furthermore, in the context of OSCC progression, there are no studies evaluating the role of agrin or Ct-agrin. To better understand the role of this protein in OSCC, we modulated the expression of agrin in aberrant keratinocytes and evaluated processes and characteristics associated with cancer progression in vitro and in vivo. Considering the potential of Ct-agrin, we identified its binding proteins in an OSCC context using mass spectrometry-

based proteomics and bioinformatics tools. Finally, we demonstrated the prognostic relevance of agrin and its protein network using publicly available cancer databases.

METHODS

Ethical statement. These procedures were in accordance with the Helsinki Declaration and the guidelines for the welfare and use of animals in cancer research [20]. This research was approved by the Institutional Animal Ethics Committee (CEUA/CNPEM), protocol #13/2015.

Theoretical assumptions. a) If the silencing of a gene inhibits the processes that are essential for malignant progression, this gene and its product could represent potential therapeutic targets. b) The disintegration of the basement membrane upon local invasion processes can release agrin processing products such as Ct-agrin [17]. Ct-agrin is a biomarker for several pathological processes. Oral cancer tumors have proteases that are capable of generating this bioactive fragment. Focusing on the dynamics of tumor progression, we believe that Ct-agrin can help explain the role of agrin in oral cancer. c) A majority of proteins interact with others in order to carry out their functions [21]. We consider that the role of a protein can be explored by knowing the function of its associated proteins.

General design. Figure 1 shows the main steps in this study. In the first experiment, we investigated whether silencing agrin interferes with tumor growth and expansion. The results in cells and mice showed that the blockage of agrin expression reduces both tumor progression and severity of the lesions. However, we did not have information on the mechanisms behind these results. We assumed that Ct-agrin could help explain these findings. Next, in order to better understand the role of agrin, we identified the protein network associated with Ct-agrin. Finally, we evaluated the clinical relevance of agrin and its network.

Subjects.

Cells. The following cell lines were used: HMK and HaCaT (normal keratinocytes), SCC-9 and SCC-25 (OSCC), HSC-3 and SCC-9-LN1 (metastatic OSCC), and HEK-293 (variable tumorigenic potential [22]. Culture conditions are summarized in the Supplement.

Mice. Age matched NOD-SCID male mice (6–weeks old) were obtained under specific pathogen-free conditions (FMUSP, São Paulo, Brazil). Animals were maintained under controlled conditions (photoperiod of 12:12 light/dark cycle at 21–24°C) with freely available food and water, in groups of 4 mice, in polycarbonate cages enriched with sterilized tissue paper.

Procedures.

Generation of agrin silenced cells. We performed agrin (isoform 1, also known as secreted agrin) silencing studies using short hairpin RNA (shRNA)-expressing vectors (Supplement). We generated the following two cell groups: agrin silenced cells (shAgrin) and non-silencing control cells (shControl). Agrin silencing was verified by real-time quantitative PCR (RT-qPCR) and western blot.

Generation of C-terminal agrin-overexpressing cells. We simulated a secreted bioactive fragment of agrin using the C-agrin$_{4,19}$-GFP construct [23] (Ct-agrin; Supplement). We used a FLAG-tagged GFP (named Ip-control) vector as a control. The Ct-agrin vector produced cytotoxicity in SCC-9 and HSC-3 cells (Supplementary Figure 1A). We solved this problem by transfecting HEK-293 cells over 48 h (Supplementary Figure 1B).

Gene expression analysis. We performed RT-qPCR. Total RNA was extracted, and cDNA was synthesized. RT-qPCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). Relative mRNA quantification was obtained with the $2^{-\Delta\Delta}$ Ct method [24] using the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as a reference (see supplement for further details).

Immunoblotting detection. We verified the presence of agrin by western blotting. Procedures for protein extraction can be reviewed in the Supplement. Cell lysate and secretome extracts were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane, which was blocked in 5% skimmed milk in TBST overnight, followed by incubation with the primary anti-agrin antibody (1:500 dilution) and the appropriate secondary antibody conjugated with horseradish peroxidase. We visualized immunoreactive bands by chemiluminescence.

Agrin and cancer-associated events.

Proliferation. We plated $1x10^4$ cells/well in 96-well plates. Proliferation rates were determined by measuring BrdU incorporation into DNA (Cell Proliferation ELISA BrdU Colorimetric, Roche Applied Science) following the manufacturer's protocol. Absorbance was measured at 450 nm with correction at 690 nm.

Migration and tumor cell invasion activity. We performed motility assays using 24-well chambers with uncoated 8-mm pore polycarbonate membranes (for migration) or 96-well chambers pre-coated with Matrigel Basement Membrane Matrix (for invasion; BD Biosciences). The chambers were rehydrated in a serum-free medium. A complete medium with 10% FBS served as a chemoattractant in the bottom chamber. Next, 7.4×10^4 (for migration) and 8×10^4 (for invasion) cells/well were incubated for 24 h. At the end of the assays, cells invading the membrane or Matrigel were stained with 1% toluidine blue/1% borax solution. The dye was eluted using 1% SDS. Absorbance was measured at 620 nm.

Cancer colony formation. We seeded 5×10^3 cells/well in six-well plates. The culture medium was changed every 2 days. After 9 days, cells were stained with 4% formaldehyde/0.005% gentian violet solution. Images were captured with an inverted microscope. We quantified colonies using ImageJ histogram (darkest) tool.

Circulating tumor cells. To simulate cancer cells in the blood or lymphatic circulation, we performed a three-dimensional tumorsphere culture. We cultured $6x10^5$ cells/dish in culture plastic wares with a non-adhesive surface, as described previously [25]. The multicellular tumorsphere area was analyzed using the ImageJ particle analysis tool.

***In vivo* tumorigenesis and aggressiveness of lesions.** We utilized an orthotopic model of OSCC. Mice were randomly divided into the following 2 groups (n=8 animals in each): HSC-3 shControl and HSC-3 shAgrin. Then, 2.5×10^5 cells/tongue in 20 μL of Matrigel were intrabuccally implanted into the right lateral portion of the tongue. Animal health was monitored daily. After 21 days, tumor aggressiveness was established by presence of ulcerations and histopathological examination with a conventional hematoxylin-eosin technique. In a blinded fashion, we evaluated the growth pattern, keratinization, cell morphology, angiogenesis, and vascular (intravascular tumor thrombus) and neural invasion.

Determination of agrin binding partners.

Immunoprecipitation. We used the secretome extract of Ct-agrin and IP-control cells. Immunoprecipitation was performed at 4°C with 2.5 µg of GFP antibody (#af4240, R&D Systems) in the presence of 30 µL of protein G-Sepharose 4 Fast Flow (GE Healthcare) for 2 h in a rocker. Sample proteins of 250 µg were added and incubated with the beads overnight at 4°C. The sepharose-bound proteins were washed with cold TBTS. Bound proteins were eluted with 4X Laemmli sample buffer at 95°C for 10 min and resolved by SDS–PAGE for subsequent western blotting (Supplementary Figure 1C).

Protein identification. From 3 biological replicates, we excised, reduced, alkylated, trypsin-digested and desalted 60 SDS-PAGE gel bands containing soluble proteins of agrin complexes according to previous protocols [26] (Supplementary Figure 1C). Tryptic digested peptides were identified in an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific Inc.) according to our previous protocols [26]. Identification of proteins was performed using MaxQuant v1.5 [27] and Perseus [28] v1.5, as described previously [29]. See Supplement for further details. For bioinformatics, we focused on proteins identified exclusively in the Ct-agrin group.

Agrin network characterization.

Cellular model validation. We used the Integrated Pathway Analysis Database for Systematic Enrichment Analysis (IPAD) [30]. IPAD was useful to validate inter-association between our identified proteins list and diseases. We considered the model validated if oral cancer appeared within the top 10 ranked diseases.

Protein-protein interactions. We assigned numeric values to all identified proteins as follows: -3 (Ip-control exclusive proteins), +3 (Ct-agrin exclusive proteins) and 1 (common proteins). Networks were visualized using the Contextual Hub Analysis Tool (CHAT app) for Cytoscape v3.4. software [31]. First, neighbor interactors were sourced from 4 databases (InnateDB-all, Mentha, IntAct, and UniProt). Then, we identified the most important centers of activity (top 20 contextual nodes, hereafter called hubs). To prioritize proteins, we used the cBio cancer genomics portal [32]. We chose those proteins that presented any alteration in a

percentage equal to or higher than 20% of the Cancer Genome Atlas HNSCC sample (TCGA 2015, n=279) [33] (Supplementary Figure 1C).

Network description. We used the STRING database [34] to retrieve the predicted interactions for the prioritized proteins. STRING v10.5 gives an association score for two interacting proteins. A high score indicates greater association confidence. We further described the proteins using QuickGO [35]. We evaluated the gene expression levels of the agrin network in agrin silenced and control cells.

Prognostic potential. We compared the gene expression levels of the agrin network in microarray data sets (OSCC vs normal tissues) using Oncomine [36]. We defined the threshold for the *P*-value and fold change as 1E-4 and 2, respectively. The rank for a gene is the median rank for that gene across each of the analyses. Additionally, we studied differential protein IHC staining in HNSCCs using protein data from the Cancer Atlas (TCGA 2015, n=279) [37]. We consulted the PROGgene online tool [38] to determine the prognostic value of the agrin network in HNSCCs. We used the GSE65858 dataset (n=269) [39] to analyze overall survival (adjusted to clinical stage).

Statistical analysis. All independent experiments were performed in triplicate. We presented the results as the mean ± standard deviation (SD). We analyzed differences between groups using Chi-Square, Student's t-test, Fisher's exact and one-way ANOVA (with post hoc Tukey) tests. PROGGene was used to create survival curves using the Cox survival analysis. In all the procedures, we used a 95% confidence level (P-value ≤ 0.05).

RESULTS

Agrin silencing suppresses cancer progression events. Most oral cancer cell lines secreted high levels of agrin (Figure 2A, right panel). We used shRNA technology to knockdown agrin expression in 3 cell lines. The knockdown efficiency of agrin shRNA was confirmed by RT-qPCR and dot blot (Figure 2B). Compared to non-target shRNA, treatment with agrin shRNA resulted in a significant decrease in cell proliferation, migration, invasion and mRNA levels of the OSCC oncogene, *EGFR*. (Figure 2C). In addition, agrin silencing suppresses the colony and tumor spheroids formation ability of OSCC cell lines (Figure 2D and E). Normal cells were unaffected by agrin silencing.

Agrin silencing reduces tumor aggressiveness. As shown in Figure 3A (right panel), mice that received agrin silenced cells (shAgrin) developed less aggressive tumors. These tumors did not have ulcers, presenting as a well-defined mass. According to histopathological examination, the shAgrin group produced tumors with few instances of vascular and nervous invasion (Figure 3B). The characteristics of tumors generated from control cells show significantly greater severity in comparison to tumors originating from agrin silenced cells. An additional panel of histological images can be seen in Supplementary Figure 2.

Agrin network. After the immunoprecipitation experiments (agrin construction is shown in 5A), the proteins contained in the immune complexes of secreted agrin were identified using mass spectrometry (Dataset, http://www.oraldiseases.org/research/agrindataset.xlsx). We validated all identifications using IPAD enrichment. As shown in Figure 5B, tongue neoplasms are in the top 5 predicted protein-disease relationships. Then, we used the CHAT app and cBio portal to prioritize some proteins (Supplementary Figure 1C). We selected the following 9 proteins: cullin-1 (CUL1), cullin-5 (CUL5), eukaryotic initiation factor 4A-II (EIF4A2), protein NDRG1 (NDRG1), polyadenylate-binding protein 1 (PABPC1), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 (RPN1), double-stranded RNA-binding protein Staufen homolog 1 (STAU1), titin (TTN), and 14-3-3 protein zeta/delta (YWHAZ). Henceforth, we referred to agrin and its 9 partners as the "agrin network". STRING analysis resulted in a network with a clustering coefficient of 0.5 (PPI enrichment *P*-value 0.037). The strategy resulted in 2 clusters (Supplemental Figure 1C). Most representative GO terms for cellular components and molecular function categories are presented in Figure 5C. Additional information can be reviewed in Dataset.

High expression of agrin network predicts adverse prognosis. Agrin partners diminished their gene expression when agrin was silenced (Figure 5D). Further, an analysis of the agrin network across 9 Oncomine data sets revealed its upregulation (Figure 5E), which is quite similar to the trend observed in our RT-qPCR data. Head and neck cancers displayed moderate to strong agrin network immunoreactivity (Figure 5F). A high expression of agrin network is associated with lower overall survival rates in patients with head and neck cancer (hazard ratio 7.6, confidence interval 1.7-33.3, *P*-value ≤ 0.05) (Figure 5G).

DISCUSSION

A class of molecules with relevant clinical potential, particularly for HNSCC, is heparan sulfate proteoglycans [40]. They can be found on the cell surface and in the ECM. Research on these molecules as participants in cancer progression is fundamental and reveals complex relationships occurring at the microenvironment, cellular and subcellular levels [41]. In this study, we show that agrin promotes the progression of oral cancer and that its protein network predicts a poor clinical prognosis.

Here, we present evidence demonstrating that the silencing of agrin leads to changes in oncogenic cell functions (proliferation, migration, invasion, and *EGFR* expression). Treatment with shRNA for agrin did not interfere with proliferation and migration functions in normal keratinocytes that were used as controls. This selectivity could open a window for therapeutic proposals.

A previous study from our group showed that agrin can mediate migration and adhesion in oral cancer [11], and we demonstrated that agrin-silenced tumor cells showed a loss of capacity for proliferation, invasion and formation of cell agglomerates. Similarly, in the context of hepatic cancer, in which agrin is overexpressed, agrin promotes proliferation, invasion and oncogenic cellular signs [8]. These invasive and proliferative phenotypes constitute fundamental biological activities for the progression of a malignant neoplasia [42], and agrin contributes to maintaining these behaviors. In addition, the tumors generated by agrin-silenced cells in the OSCC orthotopic model exhibited reduced severity, both macro- and microscopically, showing less vascular and neural invasion, which are associated with a better clinical prognosis [3,43].

We further interrogated the signaling pathways of agrin using the soluble C-terminal region of agrin once we verified the presence of cleaved or secreted agrin in oral cancer cell secretomes but not in normal cells. Combining immunoprecipitation followed by mass spectrometry-based proteomics and bioinformatics tools, we prioritized nine proteins that were involved in the agrin network, four of which were identified in the complex.

Interestingly, the network proteins were associated with specific cancer events and showed a high clustering coefficient (0.5), representing a biologically connected community, which was confirmed by changes in gene expression in OSCC cells when agrin was silenced. CUL1 and CUL5 provide a scaffold for ubiquitin ligases. They participate in the processes of ubiquitylation and neddylation, which lead to the degradation of tumor-suppressor proteins [44]. RPN1 forms part of the ubiquitin proteasome system. It is a structural component of the

proteasome [45]. EIF4A2 boosts the malignant phenotype in solid tumors [46]. NDRG1 shows high expression in oral and oropharyngeal carcinomas; however, it is associated with a low metastases rate [47]. PABPC1 can contribute to the aggressiveness of inflammatory breast carcinoma [48]. STAU1 stabilizes the mRNA in undifferentiated cells but can mediate its degradation in differentiated cells [49]. TTN stands out in rankings of genes carrying driver mutations [50,51]. Finally, YWHAZ shows high expression in patients with esophageal cancer, and it is associated with a poor clinical prognosis [52].

In hepatocellular carcinoma, recent data suggested a model where secreted agrin serves as a mechanotransduction signal to activate YAP protein in response to stiff ECM [53]. ECM stiffness (resistance to deformation) is one of the potent regulators of cell physiology such as proliferation, morphology, and migration [54]. Future research would have to evaluate whether these phenomena and molecular functions explain how agrin promotes the progression of oral cancer.

To translate the agrin network into clinical outcomes, we demonstrated that patients with HNSCC who show a high gene expression of the agrin network have a lower survival rate. To our understanding, there is no previous research showing this potential prognosis panel.

In conclusion, our results demonstrate that agrin is important for oral cancer-associated events and is a strong candidate as a therapeutic target for OSCC. In addition, agrin network panel represents a prognostic signature for oral cancer. This study deepens the understanding of the pathophysiological role of agrin in oral cancer progression.

ACKNOWLEDGMENTS

CONICYT Becas-Chile Scholarship 8540/2014 (to Rivera, C), FONDECYT 11140507 (to González-Arriagada, WA), FAPESP Grants 2009/54067-3, 2010/19278-0 and 2016/07846-0 (to Paes Leme, AF) supported this work.

AUTHOR CONTRIBUTIONS

AFPL and CR conceived this research. CR and WAGA performed the clinical sample collection and classification. CR, FSZ, CSR, CD carried out experiments. CR analysed data. CR and AFPL drafted the manuscript. All authors have read and agreed with the final version of the manuscript.

SUPPLEMENTARY MATERIAL ONLINE

- Supplement. http://www.oraldiseases.org/research/agrinsupplement.pdf.
    - Supplementary Materials and Methods.
    - Supplementary Figures 1 and 2.
    - Supplementary Figure Legends.
        - Supplementary Figure 1. Identification of C-terminal agrin partners.
        - Supplementary Figure 2. Histological features of tongue tumors.
- Dataset. http://www.oraldiseases.org/research/agrindataset.xlsx.

FIGURE CAPTIONS

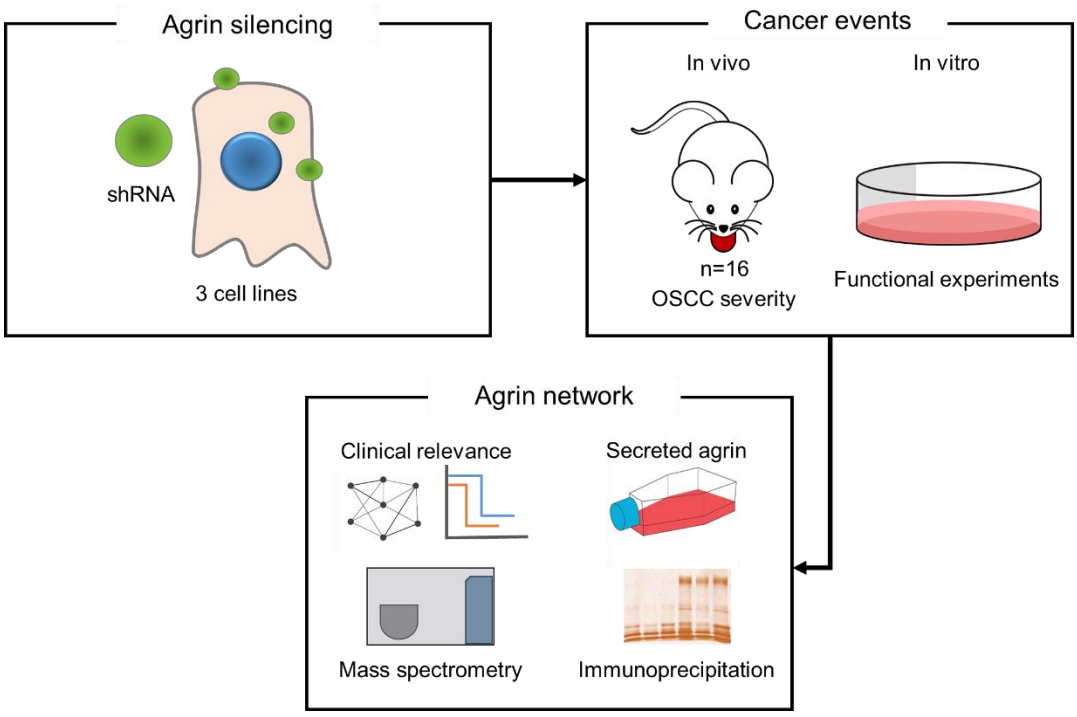

Figure 1. Schematic workflow. The effect of agrin silencing in cancer events was evaluated using both *in vitro* and *in vivo* (orthotopic model) experiments. We induced the overexpression of a secreted agrin fragment, and ligands able to bind to agrin were identified using mass spectrometry after protein immunoprecipitation. Once the agrin ligands were identified, we visualized the agrin network and evaluated its potential prognosis.

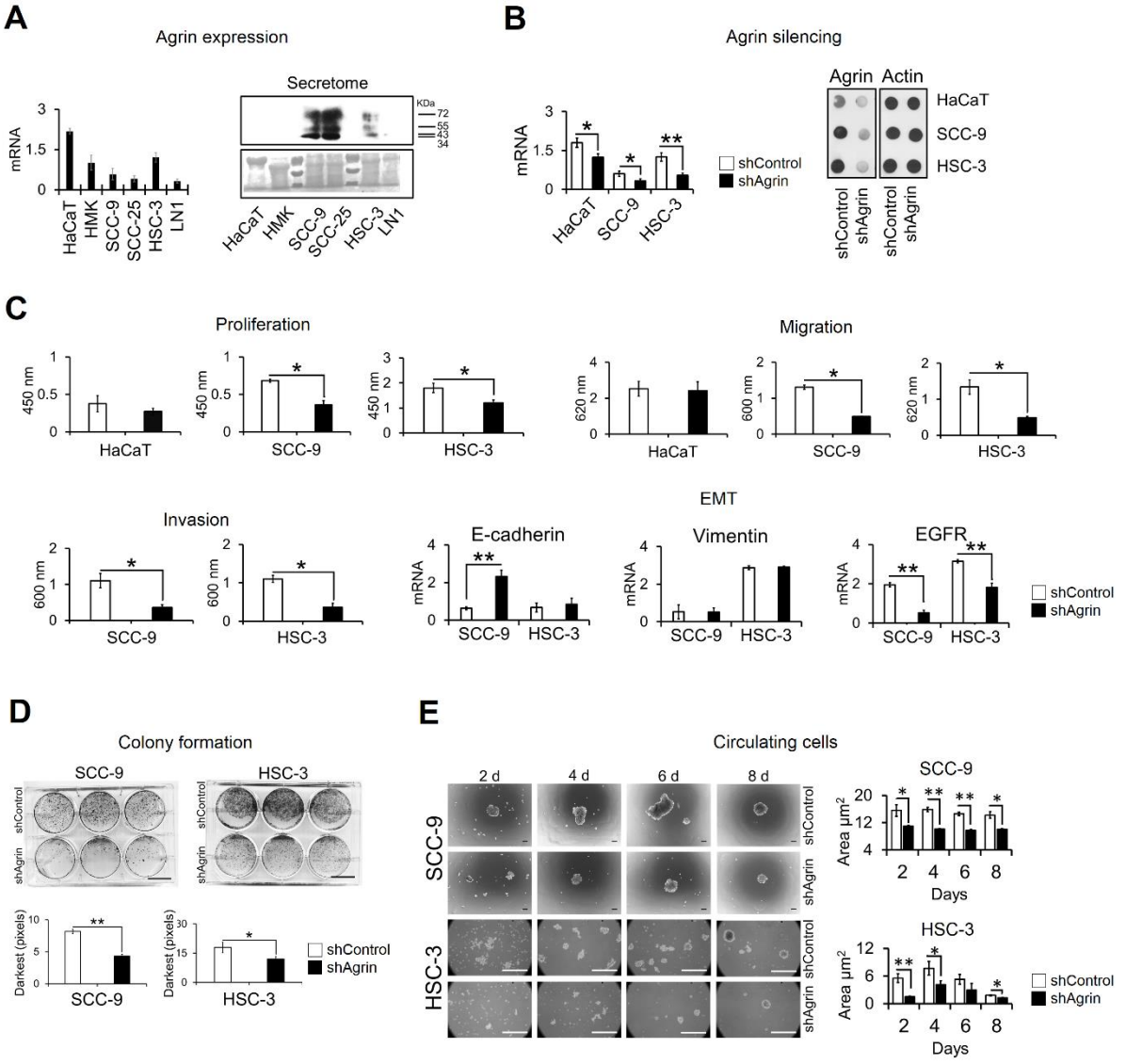

Figure 2. Agrin silencing decreased oral cancer progression, *in vitro*. (**A**) Agrin mRNA quantification in various cell lines (left panel). HMK cells were used as controls. Western blotting confirmed the presence of agrin (secretome fraction, right panel). Multiple bands may represent proteolytic cleavage products. Ponceau red was used as loading control. (**B**) Verification of agrin silencing by RT-qPCR and dot blot. Actin was used as control. (**C**) Proliferation, migration, invasion (absorbance at 450, 600 and 620 nm, respectively) and epithelial-mesenchymal transition (EMT) experiments. (**D**) Focus formation assay. Darkest intensity in millions of pixels. Scale bars, 2 cm. (**E**) Tumor sphere formation. Scale bars, 100 µm. Tumorsphere area in μm^2 (x100,000 to SCC9 and x10,000 to HSC-3). For all PCR experiments, data were normalized with *GAPDH* gene (relative quantification/GADPH, mRNA axis). Data are the means ± SD (Student's t-test, **P*-value ≤0.05, **≤0.001).

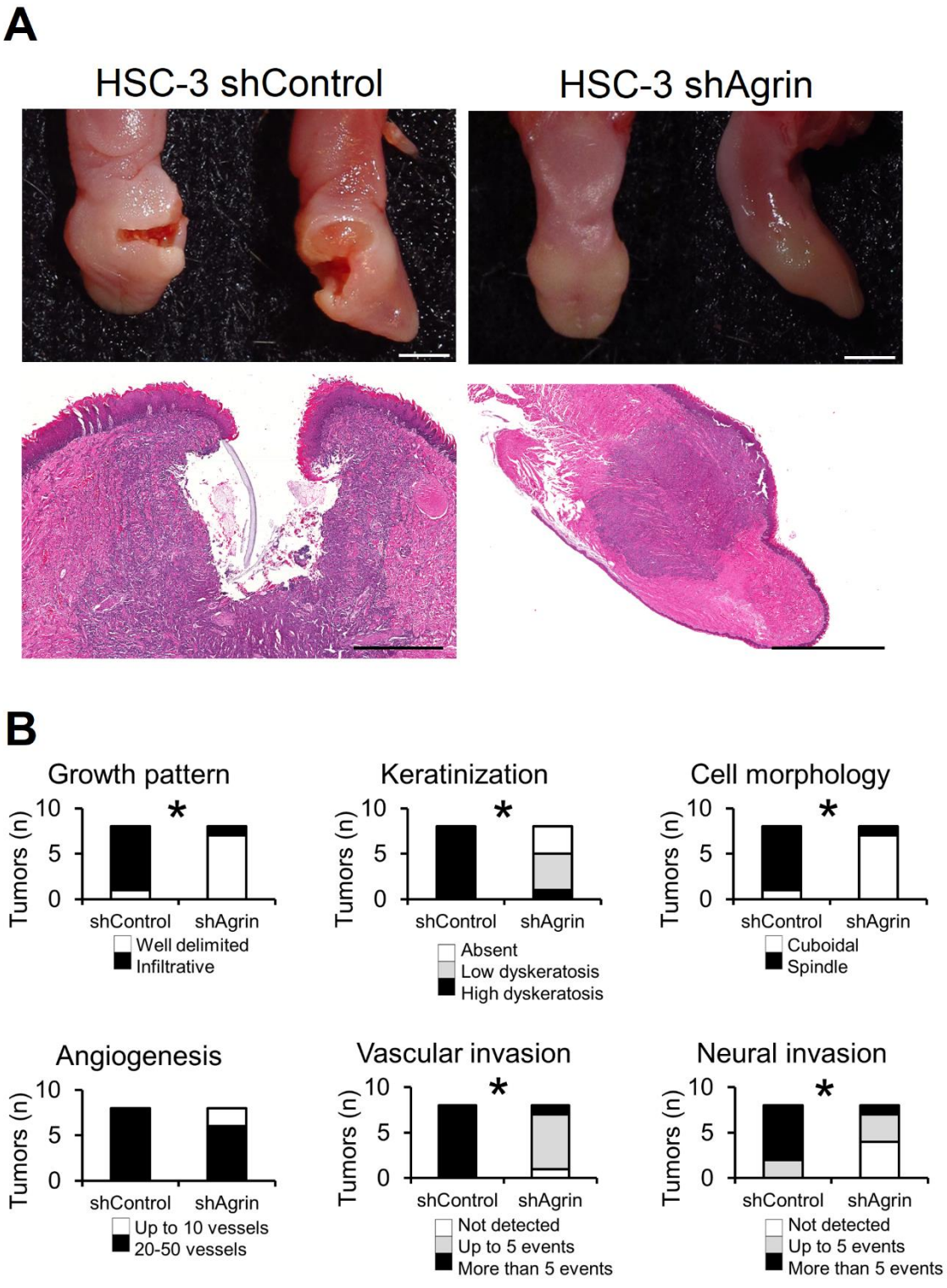

Figure 3. Agrin regulates the severity of oral cancer. (**A**) An orthotopic model of OSCC was established, inoculating HSC-3 cells into the lateral border of the tongue of NOD-SCID mice. Animals received control (shControl) or silenced (shAgrin) cells. Representative images are shown (day 21). Scale bars, 0.2 cm, 400 µm. (**B**) Histopathological characteristics of oral cancers (*P-value ≤ 0.05 Pearson's Chi-square test).

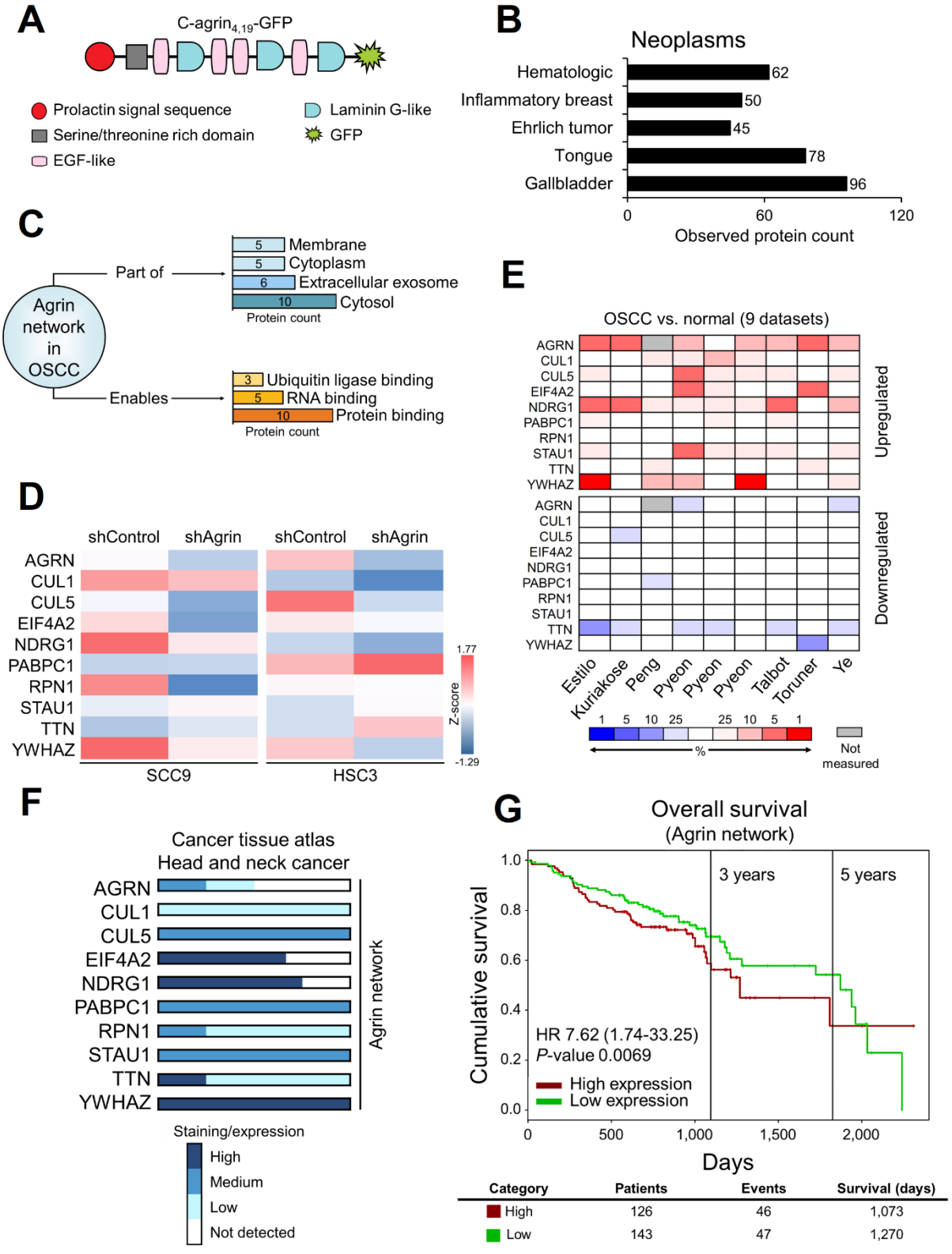

Figure 4. Agrin network represents a community with prognostic potential. (**A**) C-terminal agrin-GFP construction used as Ct-agrin in transfection experiments (PRO_0000421622). (**B**) IPAD predicted diseases from Ct-agrin ligands. Cellular model was

validated due to the presence of tongue cancers within the top 5 ranking. (**C**) Representative GO terms for the agrin network. (**D**) Agrin network gene expression is altered when agrin is silenced in OSCC cell lines. (**E**) Oncomine meta-analysis for gene expression. Color intensity equals the percentile. The names of OSCC datasets (Estilo to Ye) are written on this basis. The agrin network is overexpressed in oral cancer. (**E**) Immunohistochemical staining of agrin network, using the Cancer Atlas. (**F**) High gene expression of agrin network is associated with lower overall survival rates.

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3 DISCUSSION

Patients with OSCC generally have a poor clinical prognosis. There is a need for better prognostic tools to predict the patients' clinical course, which is important when planning the treatment (Almangush et al., 2017).

Over the years, several molecules with this potential have been proposed, which may add an additional value to the classic TNM, clinical stage or degree of differentiation. The aim of the first manuscript (published in Oral Oncology) was to identify articles that presented prognostic biomarkers for oral cancer, using a systematic review of the literature. We referred to them as "potential biomarkers," due to the absence of a sample size calculation and lack of internal/external validations.

Most of selected articles assessed proteins through immunohistochemistry in biopsies from historical archives of their clinical centers. This may be due formalin/paraffin combination is the universal method for tissue preservation (Scicchitano et al., 2009), which provided an opportunity for these retrospective studies.

The systematic review identified mostly unique biomarkers. Of the articles reviewed, only 5 used a combination of biomarkers. Today it is widely accepted that combinations of biomarkers (biomarker signatures) contain much more information than a single biomarker (Borrebaeck, 2017). The use of biomarker signatures may potentiate the discriminatory power of candidates identified by our systematic review.

In the future, widening our search for biomarkers that represent the passing from susceptibility to the establishment of a malignant neoplasm may complete our overview with respect to the prognostic biomarkers suggested for oral cancer.

Being able to demonstrate a potential clinical use is the main challenge that the prognostic markers must overcome (Kim et al., 2014). The theoretical base of the systematic review showed that the search must continue for new molecules that help us to better understand the mechanisms of oral cancer. This last point inspired the experimental studies of this thesis, which focused on the progression of oral cancer.

In recent years, the importance of tumoral stroma in the progression of cancer has been ever more evident (Sund and Kalluri, 2009). The extracellular matrix (ECM) is a fundamental part of tumoral stroma. Since malignant cells are a substantial resource that can be used as biomarkers for cancer, the ECM contains factors that may be key to malignant progression.

The ECM is composed of highly variable and dynamic components that regulate cell behavior (Oskarsson, 2013). The objective of the second manuscript was to evaluate the role of an ECM protein, agrin, in the progression of OSCC.

The silencing of agrin negatively affected the maintenance of key oncogenic phenomena for tumoral progression, which may have therapeutic implications that can be explored in the future. This confirms that the proteoglycans are important in the progression of oral neoplasms, particularly agrin.

The valuable information obtained through mass spectrometry-based proteomics made it possible to identify a group of proteins associated with agrin (agrin binding partners). The patients who presented a super high expression of the network displayed unfavorable survival curves.

Proteomics analysis is a promising approach for the discovery of biomarkers and therapeutic targets (Moghieb et al., 2013). Mass spectrometry-based proteomics is valuable in both preclinical and clinical research as it can detect multiple proteins in a biological system and correlate these patterns with health status (Maeda et al., 2015). It is the most important tool in the identification and characterization of proteins in proteomics due to the general feasibility and sensitivity of the analysis (Moghieb et al., 2013).

The pending challenge for our second study is to explore immunohistochemistry staining patterns of agrin in benign, premalignant and malignant lesions and examine the molecular functions that agrin and its binding partners represent, as well as to identify if any of these proteins can be more relevantly associated with the prognosis of patients with oral cancer.

The two studies contained in this doctoral thesis represent, firstly, our search for candidates that are associated with the clinical progression of the disease and secondly, our effort to continue opening windows, deepening the understanding of molecular mechanisms in the malignant neoplasm that is most relevant for dental surgeons, the OSCC.

4 CONCLUSION

The systematic review of the literature was useful in identifying prognostic biomarkers for oral cancer. The identified biomarkers were potential due to the lack of validation stages.

Agrin is important in the maintenance of oncogenic events associated with oral cancer, which may represent a therapeutic opportunity. Its network is a source of prognostic marker signatures for OSCC.

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[*] According to UNICAMP/FOP standards, based on the standardization of the International Committee of Medical Journal Editors - Vancouver Group. Journal abbreviations in accordance with PubMed.

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ANNEXS

Annex 1 - Approval CEUA/CNPEM Institutional Review Board.

CNPEM

Comissão de Ética no uso de Animais
CEUA/CNPEM

Certificamos que o projeto intitulado "**Role of heparan sulfate proteoglycan agrin in oral squamous cell carcinoma progression"** (protocolo n° **13**), sob responsabilidade de **Adriana Paes Leme**- que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto Homem), para fins de pesquisa científica- encontra-se de acordo com os preceitos da Lei n° 11.794, de 8 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA-CNPEM), do Centro Nacional de Pesquisa em Energia e Materiais, em reunião de 28/04/2015.

Vigência do projeto	01/01/2015 a 01/01/2016
Espécie/linhagem	Camundongo/ Balb c nude
Número de animais	32
Peso/idade	25g/ 5 semanas
Sexo	M
Origem	Biotério FMUSP

Campinas, 05 de maio de 2015

Dra. Carolina F. M. Z. Clemente
Coordenadora

Dra. Maria Carolina Scatolin do Rio
Bióloga

Annex 2 - Elsevier's policy on article sharing (Oral Oncology).

ELSEVIER

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Annex 3 - Article submission (The Journal of Pathology).

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Dear Prof. Rivera

Your manuscript entitled 'Agrin is a hub in oral cancer progression' has been successfully submitted online and will be considered for publication in The Journal of Pathology.

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